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**Suppression of Human  $\alpha$ -Globin Gene Expression Mediated by the Recombinant Aden -associated Virus 2-based Antisense Vectors**

By Selvarangan Ponnazhagan,\* Madhavi L. Nallari,\* and Arun Srivastava\*†

From the Departments of \*Medicine and †Microbiology and Immunology, Division of Hematology/Oncology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5120

**Summary**

We sought to investigate the usefulness of the adeno-associated virus 2 (AAV)-based vectors to suppress the excess production of the human  $\alpha$ -globin gene product towards developing a treatment modality for  $\beta$ -thalassemia since accumulation of free  $\alpha$ -globin reduces the lifespan of red blood cells in these patients. We constructed recombinant AAV virions containing the human  $\alpha$ -globin gene sequences in antisense orientation driven by the herpesvirus thymidine kinase (TK) promoter, the SV40 early gene promoter, and the human  $\alpha$ -globin gene promoter, respectively, as well as a bacterial gene for resistance to neomycin (*neo*<sup>R</sup>) as a selectable marker. These recombinant virions were used to infect a human erythroleukemia cell line (K562) that expresses high levels of  $\alpha$ -globin mRNA. Clonal populations of *neo*<sup>R</sup> cells were obtained after selection with the drug G418, a neomycin analogue. Total genomic DNA samples isolated from these cells were analyzed on Southern blots to document stable integration of the transduced *neo* and  $\alpha$ -globin genes. Total cellular RNA samples isolated from mock-infected and recombinant virus-infected cultures were also analyzed by Northern blots. Whereas the TK promoter-driven antisense  $\alpha$ -globin sequences showed no inhibition of expression of the endogenous  $\alpha$ -globin gene, the SV40 promoter and the  $\alpha$ -globin gene promoter-driven antisense  $\alpha$ -globin sequences suppressed the expression of this constitutively over-expressed gene by approximately 29 and 91%, respectively, at the transcriptional level. These studies suggest the feasibility of utilizing the AAV-based antisense gene transfer approach in the potential treatment of  $\beta$ -thalassemia.

Adeno-associated virus 2 (AAV) is a nonpathogenic human parvovirus that requires coinfection with either adeno-virus, herpesvirus, or vaccinia virus for its optimal growth and replication (1). In the absence of a helper virus, the AAV genome integrates into chromosomal DNA in a site-specific manner on chromosome 19 in human cells (2-4). The AAV genome is a single-stranded DNA containing identical inverted terminal repeats (ITRs) that act as the sole *cis*-acting elements for the viral DNA replication (5-7). Several AAV-based recombinant virions have been constructed by replacing the entire viral genome with a foreign DNA between the two ITRs, and have been shown to be functionally active upon infection of a variety of established cell lines and primary cell cultures in vitro (8-10).

The nonpathogenic nature of AAV, coupled with the remarkable site specificity of integration, has added to its desirable features as a vector for gene therapy (8-13). In the current study, we investigated the potential application of the AAV-based vector system as a treatment modality for

$\beta$ -thalassemia, a heritable disorder characterized by defective synthesis of  $\beta$ -globin chains. In the homozygous condition,  $\beta$ -thalassemia is also characterized by accumulation of excessive free  $\alpha$ -globin chains which results in premature death of erythroid progenitor cells before they reach the reticulocyte stage (14). This imbalanced production of globin chains results in red cell damage shortening their lifespan. A mechanism that could control the excess production of free  $\alpha$ -globin chains would be expected to largely benefit in the amelioration of clinical severity of this disease.

The strategy we employed was based on suppression of the augmented  $\alpha$ -globin levels at the transcriptional stage through the production of antisense  $\alpha$ -globin RNA sequences mediated by AAV. We observed significant inhibition of expression of the endogenous  $\alpha$ -globin gene in K562 cells upon infection with recombinant AAV virions harboring the antisense human  $\alpha$ -globin DNA sequences driven either by the SV40 early promoter or the human  $\alpha$ -globin gene promoter. We suggest that the AAV-based vector system may prove useful

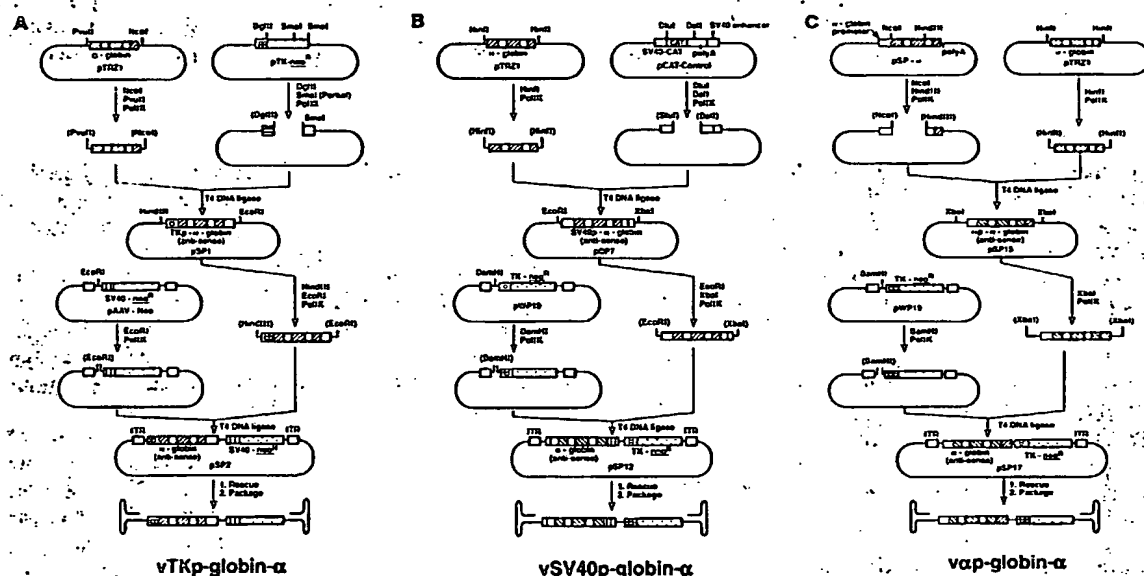
in the potential gene therapy of human hemoglobinopathies in general, and  $\beta$ -thalassemia in particular.

## Materials and Methods

**Plasmids, Viruses, and Cells.** Recombinant clones of AAV plasmids have been described (15, 16), and were obtained from Dr. R. J. Samulski (University of North Carolina, Chapel Hill, NC). Two recombinant AAV plasmids containing the *neo<sup>R</sup>* gene driven by either the SV40 promoter (pAAV-Neo), or the TK promoter (pWP-19), have also been described (17, 18). A recombinant plasmid containing the genomic copy of human  $\alpha$ -globin gene (pTRZ1) was kindly provided by Dr. George Stamatoyannopoulos (University of Washington, Seattle, WA). A recombinant plasmid containing the SV40 promoter-driven chloramphenicol acetyl transferase (CAT) gene (pCAT-control) was purchased from Promega Biotech (Madison, WI). Human adeno-associated virus 2 (AAV-2H), and human adenovirus 2 (Ad2) viral stocks were provided, respectively, by Dr. K. I. Berns, (Cornell University Medical College, New York) and Dr. K. H. Fife (Indiana University School of Medicine, Indianapolis, IN), and were grown and maintained as previously described (19-21). The human erythroleukemia cell line, K562, was obtained from the American Type Culture Collection (Rockville, MD), and maintained in IMDM supplemented with 10% FBS.

**Construction of Recombinant Plasmids and Viruses.** The overall strategy for the construction of the three different recombinant AAV plasmids is illustrated in Fig. 1. Plasmid pSP-2, containing the thymidine kinase (TK) promoter-driven human  $\alpha$ -globin gene sequence in antisense orientation (Fig. 1 A) was constructed as follows: the  $\alpha$ -globin fragment was isolated from plasmid pTRZ1 using restriction enzymes *Nco*I and *Pvu*II, and cloned between

*Bgl*II and *Sma*I sites of plasmid pTK-Neo after treatment with *Pol*k by the standard methods described in Sambrook et al. (22) to yield a plasmid, designated pSP-1, which also contained the TK polyadenylation (poly A) signal sequence. A DNA fragment containing the TK promoter-driven antisense  $\alpha$ -globin gene was excised from plasmid pSP-1 using *Eco*RI and *Hind*III and cloned at the *Eco*RI site of plasmid pAAV-Neo after treatment with *Pol*k to yield a plasmid, designated pSP-2. This plasmid contains the antisense  $\alpha$ -globin sequences driven by the TK promoter, and the *neo<sup>R</sup>* gene driven by the SV40 promoter. Plasmid pSP-12, containing the SV40 promoter-driven  $\alpha$ -globin gene in antisense orientation, and the TK promoter-driven *neo<sup>R</sup>* gene (Fig. 1 B), was constructed by replacing the CAT coding sequences in pCAT-control by the  $\alpha$ -globin sequences in antisense orientation to generate a plasmid, designated pSP-7, in which the SV40 poly A signal was retained at the 3' end. Further, the SV40 promoter-driven antisense  $\alpha$ -globin sequences were isolated from pSP-7 using *Eco*RI and *Xba*I, and cloned at the *Bam*HI site of pWP-19 after treatment with *Pol*k. The resulting plasmid, designated pSP-12, contains SV40 promoter-driven antisense  $\alpha$ -globin sequences and the TK promoter-driven *neo<sup>R</sup>* gene sequences flanked by the AAV-ITRs. Plasmid pSP-17 was constructed with the human  $\alpha$ -globin gene promoter-driven antisense  $\alpha$ -globin sequences, and the TK promoter-driven *neo<sup>R</sup>* (Fig. 1 C). Plasmid pSP- $\alpha$ , containing the entire coding region as well as the promoter region of the human  $\alpha$ -globin sequences, was digested with *Nco*I and *Hind*III to remove the coding region of the  $\alpha$ -globin coding sequence, and a 1.0-kb DNA fragment isolated from *Hinf*I-digestion of plasmid pTRZ1 that contained the  $\alpha$ -globin coding region was cloned in antisense orientation after treatment with *Pol*k to yield a plasmid, designated pSP-15. The  $\alpha$ -globin poly A sequence was retained in pSP-15. The  $\alpha$ -globin promoter-driven antisense  $\alpha$ -globin sequences were further cloned



**Figure 1.** Construction of antisense human  $\alpha$ -globin gene-containing recombinant AAV plasmids and virions. The overall strategy for construction of the recombinant AAV genomes containing antisense  $\alpha$ -globin sequences driven by the TK promoter (vTKp-globin- $\alpha$ ; A), the SV40 promoter (vSV40p-globin- $\alpha$ ; B), and the  $\alpha$ -globin promoter (vap-globin- $\alpha$ ; C), is described under Materials and Methods.

at the BamHI site of plasmid pWP-19 after treatment with Pollk to generate a recombinant plasmid, designated pSP-17.

All three recombinant AAV genomes were rescued from their respective plasmids, packaged into mature AAV virions, as previously described (23), and designated vTKp-globin- $\alpha$ , vSV40p-globin- $\alpha$ , and v $\alpha$ p-globin- $\alpha$ , respectively. The viral titers were determined on quantitative DNA slot blots as previously described (24).

**Recombinant Virus Infections and Selection of *neo*<sup>R</sup> Colonies.** K562 cells were infected with the recombinant viruses at a multiplicity of infection (m.o.i.) of 1 in 0.5 ml of IMDM. Infection was allowed to proceed at 37°C for 2 h after which the cells were plated in 10 ml IMDM supplemented with 10% FBS and grown at 37°C. 48 h post-infection (p.i.), the drug G418 was added to the medium at a final active concentration of 400  $\mu$ g/ml, and the cultures were maintained for at least 2 wk before selecting the *neo*<sup>R</sup> clonal cell populations. The *neo*<sup>R</sup> colonies were propagated in IMDM supplemented with 10% FBS and 400  $\mu$ g/ml G418.

**Southern Blot Analysis for Viral Integration.** Total genomic DNA samples isolated from mock-infected, or the recombinant virus-infected, *neo*<sup>R</sup> clones of K562 cells were subjected to digestion with a variety of restriction endonucleases and analyzed on Southern blots as previously described (25). <sup>32</sup>P-labeled  $\alpha$ -globin- and neomycin-specific DNA probes were used to hybridize to the filters and autoradiographed.

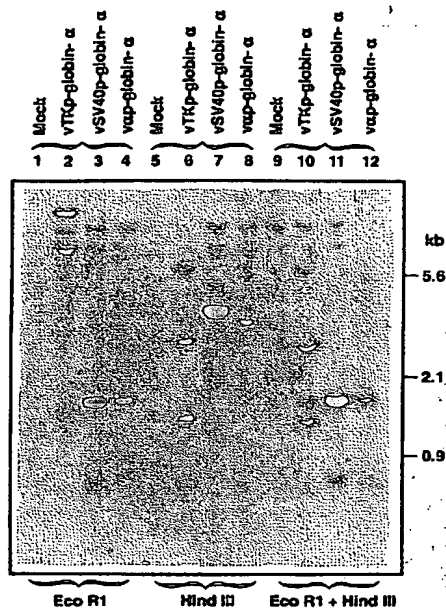
**Northern Blot Analysis of Expression of the Endogenous  $\alpha$ -Globin Gene.** Total cellular RNA was isolated from the recombinant virus-infected *neo*<sup>R</sup> colonies of K562 cells as well as from mock-infected K562 cells following standard methods (26). Northern blots of equivalent amounts of different RNA samples were probed with <sup>32</sup>P-labeled  $\alpha$ -globin-,  $\beta$ -actin-, and neomycin-specific DNA probes. The levels of expression of the endogenous  $\alpha$ -globin RNAs were quantitated by scanning the autoradiograms using a video densitometer (model 620; Bio-Rad Laboratories, Richmond, CA).

## Results

**Successful Transduction of K562 Cells with the Recombinant AAV Vectors.** The recombinant AAV genomes we constructed in these studies were ~5.0 kb in length, and could thus be successfully packaged into mature virions to generate vTKp-globin- $\alpha$ , vSV40p-globin- $\alpha$ , and v $\alpha$ p-globin- $\alpha$  recombinant AAV virions, respectively (Fig. 1). Since the recombinant viral genomes also contained a *neo*<sup>R</sup> selectable-marker gene, upon infection of K562 cells with these virions, G418-resistant cell populations could be readily obtained. These clonal cell populations for each recombinant virus were pooled and their genomic DNA was analyzed on Southern blots as described under Materials and Methods. A representative Southern blot probed with a *neo*-specific DNA probe is shown in Fig. 2.

As is evident, none of the DNA samples isolated from mock-infected K562 cells contained specific *neo*-hybridizing bands. However, in DNA samples isolated from each of the recombinant virus-infected cell populations, the probe detected several distinct hybridizing fragments suggesting stable integration of the recombinant viral genomes into the chromosomal DNA. Similar results were obtained when an  $\alpha$ -globin-specific DNA was used as a probe except for detection of the endogenous alleles (data not shown).

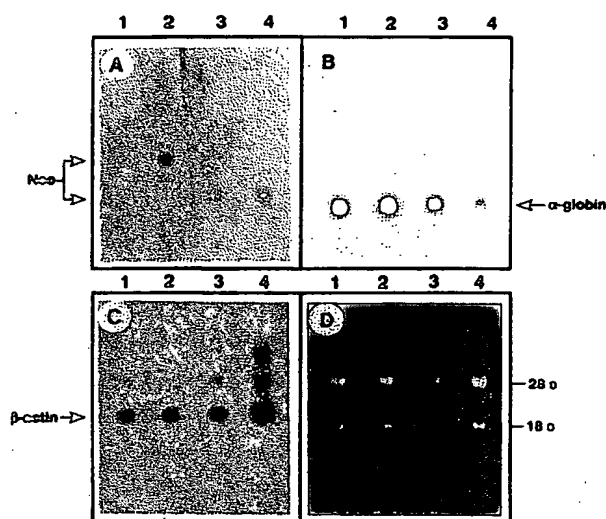
**Expression Analysis of the Endogenous  $\alpha$ -globin Gene.** It was



**Figure 2.** Southern blot analysis of stable transduction of the recombinant AAV genomes in K562 cells. Total genomic DNA samples isolated from K562 cells after mock-infection (lanes 1, 5, 9), or transduction with vTKp-globin- $\alpha$  (lanes 2, 6, 10), vSV40p-globin- $\alpha$  (lanes 3, 7, 11), and v $\alpha$ p-globin- $\alpha$  (lanes 4, 8, 12), were cleaved with the indicated restriction endonucleases and analyzed on Southern blots using a *neo*-specific DNA probe as described under Materials and Methods. HindIII-cleaved PM2 DNA fragments were coelectrophoresed as size-markers.

next of interest to determine whether the transduced antisense  $\alpha$ -globin sequences modulated the levels of expression of the constitutively expressed  $\alpha$ -globin gene in K562 cells. Total RNA samples isolated from mock-infected, or the recombinant virus-infected cells were analyzed on Northern blots as described under Materials and Methods. Such a blot is depicted in Fig. 3.

It is evident that when a *neo*-specific DNA was used as probe (Fig. 3 A), no hybridization was detected in mock-infected K562 cells (Fig. 3, lane 1), whereas the probe detected specific RNA transcripts in the three recombinant virus-infected cultures (Fig. 3, lanes 2-4). The sizes of the transcripts correspond to their respective genomes. When the same blot was probed with an  $\alpha$ -globin probe (Fig. 3 B), mock-infected (Fig. 3, lane 1) as well as vTKp-globin- $\alpha$  virus-infected (Fig. 3, lane 2) K562 showed abundant expression of the endogenous  $\alpha$ -globin gene. However, cell populations transduced with vSV40p-globin- $\alpha$  (Fig. 3, lane 3) and v $\alpha$ p-globin- $\alpha$  (Fig. 3, lane 4) virions showed a clear suppression of expression of the endogenous gene. Densitometric analyses of the hybridization signals revealed the extent of this suppression to be ~29% with vSV40p-globin- $\alpha$ , and ~91% with v $\alpha$ p-globin- $\alpha$  recombinant virions with reference to the levels of  $\beta$ -actin mRNA (Fig. 3 C). These levels also corresponded well with ethidium bromide-induced fluorescence intensities



**Figure 3.** Northern blot analysis of expression of the endogenous human  $\alpha$ -globin gene in K562 cells. Total RNA samples isolated from mock-infected (lane 1), vTKp-globin- $\alpha$  virus-infected (lane 2), vSV40p-globin- $\alpha$  virus-infected (lane 3), and v $\alpha$ p-globin- $\alpha$  virus-infected (lane 4) K562 cells were analyzed on Northern blots using the following DNA probes: *neo* (A),  $\alpha$ -globin (B), and  $\beta$ -actin (C) as described under Materials and Methods. Ethidium bromide-induced fluorescence of 28s and 18s ribosomal RNAs is also shown (D).

of 28s and 18s ribosomal RNA bands in these cell populations (Fig. 3 D). We also noted that expression of the *neo*-specific RNA transcripts was significantly lower with the TK promoter than with the SV40 promoter (Fig. 3 A) indicating the relative inefficiency of the TK promoter function compared with that of the SV40 promoter in K562 cells.

These data suggest that it may indeed be feasible to employ the AAV-based antisense vectors to modulate the expression of the constitutively over-expressed  $\alpha$ -globin gene in human hematopoietic cells.

## Discussion

The potential application of the AAV-based vector system for the purpose of gene therapy in humans is gaining attention (8–13). That the AAV-based vectors may prove to be a useful alternative to the more commonly used retrovirus-based vectors, stems from the fact that AAV is a nonpathogenic virus whereas retroviruses are intimately associated with neoplastic events (27). AAV has thus far not been shown to be associated with any malignant disease (28), and interestingly, AAV has been shown to possess anti-oncogenic properties both in vitro and in vivo (29–32).

Of the several human diseases amenable to genetic correction, those affecting the hematopoietic system appear to be potential candidates for gene therapy. In the case of  $\beta$ -tha-

lassemia, which is characterized by the defective  $\beta$ -globin production, a mechanism to deliver a functional  $\beta$ -globin gene would be a preferred form of treatment. Indeed, in our recent studies with the recombinant AAV vector system, we have documented successful transduction and erythroid cell-specific expression of a normal human  $\beta$ -globin gene in human hematopoietic cells in vitro (32a). It is also noteworthy that Walsh et al. (8) have recently described the construction of, and in vitro expression from, a recombinant AAV- $\gamma$ -globin vector.

A second line of therapeutic measure that could ameliorate the severity of this disease would be to suppress the accumulation of excessive levels of the  $\alpha$ -globin chains in red blood cells which leads to their premature destruction. The antisense strategy that we employed in our present studies might prove as a useful mode of treatment for such a defect. The extent of suppression was highest with the  $\alpha$ -globin gene promoter-driven antisense sequences. This probably suggests the maximal efficiency of a natural promoter in transcriptional activation by *trans*-acting factors compared with the heterologous promoters. The mechanism of antisense RNA inhibiting the expression of a target gene is not clearly understood. Yet, in principle, the antisense RNA could act at the following level(s): transcription, mRNA processing, transport out of the nucleus, translation, and/or general stability of the mRNA (33). A cogent example of near-total suppression in vitro of HIV-1 replication mediated by an antisense AAV vector has recently been provided by Chatterjee et al. (9). In our studies, no attempts were made to control the levels of antisense RNA production, but we presume that most of the antisense RNA transcripts that were generated were utilized in hybridization with the complementary target  $\alpha$ -globin mRNA. Using strand-specific RNA probes, we could not detect any significantly free antisense transcripts in K562 cells transduced with the recombinant AAV virions, yet abundant expression of these transcripts was evident in transduced HeLa cells as determined by Northern blot analysis (data not shown).

Although we were able to achieve high-level suppression of expression of the endogenous  $\alpha$ -globin gene in clonal populations of relatively homogeneous K562 cells, it may be undesirable to completely block the expression of the required basal amounts of the endogenous  $\alpha$ -globin gene in primary human hematopoietic cells by over-producing the antisense  $\alpha$ -globin RNA transcripts. However, given the heterogeneous nature of the primary human hematopoietic cells, we believe this unlikely to be the case. Whether this strategy with primary cultures will prove to be effective in vitro remains to be determined. It also remains to be ascertained whether this treatment modality will be safe and efficacious in an animal model in vivo, before its potential use in human gene therapy. This form of gene transfer using antisense RNA production may also prove to be useful in a wide variety of disease conditions that are characterized by undesirable over-expression of endogenous genes as well as that from infectious agents.

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Address correspondence to Dr. Arun Srivastava, Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Drive, MS-255, Indianapolis, IN 46202-5120.

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